Chromosome aberrations in adenomas of the colon. Proof of trisomy 7 in tumor cells by combined interphase cytogenetics and immunocytochemistry

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CHROMOSOME ABERRATIONS IN ADENOMAS OF THE COLON. PROOF OF TRISOMY 7 IN TUMOR CELLS BY COMBINED INTERPHASE CYTOGENETICS AND IMMUNOCYTOCHEMISTRY

J. HERBERGS[1,4], A.P. DE BRUÏNE[1], P.T.J. MARX[1], M.I.J. VALLINGA[2], R.W. STOCKBRÜGGER[3], F.C.S. RAMAEKERS[2], J.W. ARENDS[1] and A.H.N. HOPMAN[2]

[1]*Department of Pathology, University Hospital, Maastricht;* [2]*Department of Molecular Cell Biology and Genetics, University of Limburg, Maastricht; and* [3]*Department of Internal Medicine, University Hospital, Maastricht, The Netherlands.*

Thirty-five colon adenomas from 26 patients were analyzed with centromeric probes for chromosomes 1, 7, 17, X and Y in order to study numerical aberrations, chromosome imbalances, aneuploidy and tetraploidization. The fluorescent *in situ* hybridization (FISH) technique was applied to single-cell suspensions and a combination of FISH and immunocytochemistry (ICC) was employed to identify the cell type under study. Trisomy of chromosome 7 was detected in 37% of the cases. In 7 out of 13 cases this aberration was combined with abnormalities of one or 2 of the other investigated chromosomes. No correlation could be demonstrated between any of the detected chromosomal aberrations and size, localization or degree of epithelial dysplasia. With the combined FISH/ICC procedure, the abnormal cells were shown to be of epithelial rather than of stromal origin. Our data indicate that trisomy 7 is a common chromosome aberration in the epithelial component of colon adenomas.

Non-random chromosomal abnormalities have been observed in many types of human solid tumors (Sandberg, 1990). Most information on the genomic changes is acquired by karyotyping analysis of solid tumor cultures, but in recent years the fluorescent *in situ* hybridization (FISH) procedure has been used for targeted analyses of numerical chromosome abnormalities within interphase nuclei of (pre)malignancies.

In many different tumor types, including colorectal adenocarcinomas (Muleris *et al.*, 1990; Bardi *et al.*, 1993), trisomy of chromosome 7 is a frequently found abnormality. In many of these neoplasms, trisomy 7 was the sole chromosomal aberration detected and was therefore interpreted as a primary change in cancer development. Recent studies, however, suggest that trisomy 7 does not occur in the tumor cells, but in non-malignant stromal cells. Cytogenetic analyses of short-term cultures from colorectal adenocarcinomas (Bardi *et al.*, 1991) showed trisomy of chromosome 7 in fibroblast-like cells. Trisomy 7 was also found by cytogenetic analyses of short-term cultures of tumor-surrounding kidney tissue (Casalone *et al.*, 1992) and from studies using a combination of FISH and immunocytochemistry (ICC) to characterize subpopulations of tumor-infiltrating lymphocytes in kidney tumors and apparently normal kidney tissue (Dal Cin *et al.*, 1992).

Additional evidence supporting this notion is the presence of trisomy 7 in some non-malignant conditions, as in non-neoplastic human brain tissue (Heim *et al.*, 1989) and normal lung tissue (Lee *et al.*, 1987). Furthermore, trisomy 7 was detected by cytogenetic analyses of short-term cultures in precursors of malignant tumors, as in several reports concerning colonic adenomas (Williams *et al.*, 1992; Longy *et al.*, 1993).

To detect a possible role of trisomy 7 in cancer development and the cell type involved, we analyzed colonic adenomas for the occurrence of trisomy 7 and characterized the aberrant cells. For this purpose, we performed interphase cytogenetics using the FISH procedure to detect chromosome 7 aberrations in combination with the immunocytochemical detection of cytokeratin 18, to identify the cell type. Furthermore, as an indicator of overall chromosomal ploidy, we used centromeric DNA probes for chromosome 1, because numerical aberrations for this chromosome are rarely found in colorectal adenomas and adenocarcinomas (Muleris *et al.*, 1990). The centromeric probe for chromosome 17 was used since loss of chromosome 17 has been reported by other investigators in colorectal adenocarcinomas (Muleris *et al.*, 1990; Bardi *et al.*, 1993), and the sex chromosomes X and Y were detected since they are frequently involved in early stages of malignant transformation in various tumor types (Sandberg, 1990).

MATERIAL AND METHODS

Tissues

Fresh tissue of polyps from 28 patients was obtained after endoscopy and small blocks (maximum size 3 cm) were snap-frozen in liquid nitrogen. Table I summarizes the clinical data of the patients and the relevant histological data of the tissues. In one case (case 18), 7 adenomas were found.

In addition, 10 samples of normal colonic epithelium were obtained after endoscopy and analyzed in parallel with the adenomas.

Sample preparation

Fresh tissue of 38 polyps from 28 patients was divided into 2 parts, one for histopathology and one for FISH. For FISH we used single-cell suspensions, paraffin-embedded tissue, and frozen tissue. Single-cell suspensions were prepared by mincing tissue blocks of fresh adenomas. For isolation of epithelial cells the minced tissue blocks were treated with PBS containing 1 mM ethylenediamine-tetraacetic acid (EDTA; Merck, Darmstadt, Germany), 1 mM ethyleneglycol-bis-(B-amino ethyl ether)-N,N,N′, N′-tetraacetic acid (EGTA; Sigma, St Louis, MO) and 0.5 mM dithiothreitol (DTT; Sigma). The cells were fixed in 70% ethanol at −20°C (Whitehead *et al.*, 1987).

Tumor cell processing

Five microliters of the single-cell suspensions were dropped onto poly-L-lysine (MW 150,000; Sigma) coated slides and air-dried. A proteolytic digestion step was performed with pepsin from porcine stomach mucosa (2,500–3,500 units per mg protein; Sigma) at a concentration of 100 μg/ml in 0.01 N HCl for 20 min at 37°C. After subsequent dip washes in double-distilled water (5 times) and PBS (5 times), the nuclei were post-fixed in 1% formaldehyde in PBS for 20 min at 4°C. The slides were subsequently washed in PBS (5 times) and double-distilled water (5 times), and equilibrated in 60% formamide-2×SSC (Standard Saline Citrate; Merck), pH 7.0, for 5 min at room temperature.

[4]To whom correspondence and reprint requests should be sent, at the Department of Pathology, University Hospital Maastricht, P.O. Box 616, 6200 MD Maastricht, The Netherlands. Fax: 31-43-670948.

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DNA probes

For detection of the target sequences on chromosomes 1, 7, 17, X and Y, the plasmid probes were used (Willard and Waye, 1987). The probes were labeled by nick-translation with biotin 11-dUTP (Sigma) according to the supplier's instructions. The probes were hybridized in 60% formamide-2×SSC, 10% dextran sulfate (Sigma) and 1 μg/μl salmon sperm DNA (Sigma) as carrier DNA, at a probe concentration of 2 to 6 ng/μl hybridization mixture. Under these stringency conditions, hybridization to minor binding sites was avoided.

TABLE I – CLASSIFICATION OF ADENOMAS ACCORDING TO AGE AND SEX, SIZE, GROWTH PATTERN AND DEGREE OF DYSPLASIA

Case number	Age/sex	Diameter (mm)	Growth pattern	Degree of dysplasia[1]
1	70/F	12	tubular	++
2a	64/F	7	tubular	++
2b	64/F	9	villous	++
3	66/F	24	tubular	+++
4	53/M	21	tubular	++
5	55/F	17	tubular	++
6	83/F	7	tubulovillous	++
7	75/M	8	tubular	++
8	79/F	16	tubulovillous	+++
9	38/M	21	hyperplastic	ND
10	78/M	8	tubular	++
11	67/M	13	tubular	++
12	92/M	10	tubulovillous	++
13	60/M	33	tubular	+++
14	46/F	22	tubular	++
15	44/F	8	tubular	+
16	50/M	13	villous	+++
17	50/M	13	tubular	++
18a	55/M	4	tubular	++
18b	55/M	12	tubular	++
18c	55/M	7	tubular	++
18d	55/M	12	tubulovillous	++
18e	55/M	10	tubular	+++
18f	55/M	ND	tubular	++
18g	55/M	4	tubular	++
19	78/M	8	tubular	++
20	10/F	30	hyperplastic	ND
21	71/F	15	tubular	+++
22	68/M	16	tubular	+
23	70/M	12	tubular	+
24	M	ND	tubular	+
25a	73/M	14	tubular	+
25b	73/M	9	tubular	++
26	61/M	8	tubular	++
27a	79/M	7	tubulovillous	++
27b	79/M	11	tubular	++
28a	55/M	8	hyperplastic	ND
28b	55/M	13	tubular	++

[1]Degree of dysplasia: + = mild, ++ = moderate, +++ = severe; ND not done.

Fluorescence in situ hybridization

Ten microliters of the hybridization mixture were added to the slides under a coverslip (18 × 18 mm). Denaturation was performed at 80°C on a heating plate in a moist chamber for 4 min. Hybridization was then performed overnight at 37°C. Next, the slides were washed twice for 5 min in 60% formamide-2×SSC, pH 7.0, at 42°C and subsequently twice in 2×SSC, pH 7.0, for 5 min at 42°C and in the same buffer for 5 min at room temperature. Immunochemical detection was performed as previously described (Pinkel *et al.*, 1988). Nuclei were counterstained with propidium iodide (PI, Sigma) or with 4',6-diamidino-2-phenylindole (DAPI, Sigma). Evaluation of the preparations was performed by counting approximately 200 nuclei per slide, according to criteria described earlier (Hopman *et al.*, 1988).

Combining ICC and FISH

Ten microliters of the single-cell suspensions were brought to 200 μl 70% ethanol and cytospun (5 min, 1,200 rpm) onto poly-L-lysine-coated slides, air-dried and washed in PBS. The monoclonal antibody RCK 106, directed against cytokeratin 18, was used to select for epithelial cells (Ramaekers *et al.*, 1987). After washing with PBS, incubation with RAM-AP (1:80 in 2% normal goat serum, PBS-Tween 0.05%) and another washing step with PBS, the APase activity was demonstrated using a fluorescence azo-dye coupling technique. We used a stain solution containing 4 ml of 0.2 M Tris/HCl buffer, pH 8.5, with 5% polyvinyl alcohol (PVA) (40 kDa, Sigma), 1 mg of naphtol-ASMX-phosphate (Sigma) in 250 μl buffer without PVA and 5 mg of fast-red-TR salt (Sigma) in 750 μl buffer without PVA (Speel *et al.*, 1992). This staining solution was then gently mixed and used immediately. The slides were incubated with 100 μl staining solution under a coverslip for 30 min at room temperature and then washed 3 times with PBS for 5 min at room temperature and air-dried. Next, FISH was performed as described above, using the plasmid probe for the centromeric region of chromosome 7.

RESULTS

Isolation of epithelial cells

To validate the procedure for isolation of cells from adenomas (Whitehead *et al.*, 1987), which has been reported to yield a suspension of mainly epithelial cells, we performed immunocytochemical staining procedures for cytokeratin 18 with RCK 106 (Ramaekers *et al.*, 1987) on all suspensions made, in order to detect epithelial cells exclusively. In all these cases approximately 80% of the cells in suspension were cytokeratin-18-positive. The remaining cytokeratin-18-negative cells comprised the stromal and inflammatory cells.

TABLE II – NUMERICAL ABERRATIONS OF CHROMOSOMES 1, 7, 17, X AND Y IN COLONIC ADENOMAS

Case number	Numerical chromosome aberrations				
	Chr. 1	Chr. 7	Chr. 17	Chr. X	Chr. Y
4		trisomy			
5		trisomy			
13		trisomy			
14	trisomy	tri/tetrasomy	trisomy	tri/tetrasomy	
15		trisomy	trisomy	trisomy	
16					disomy
18a		trisomy			
18b		trisomy	trisomy		
18d	trisomy	trisomy	trisomy	disomy	disomy
18f		trisomy			
19		trisomy			
21	mono/trisomy	tri/tetrasomy			
22	trisomy	trisomy			
27a	trisomy	tri/tetrasomy	tri/tetrasomy	disomy	disomy

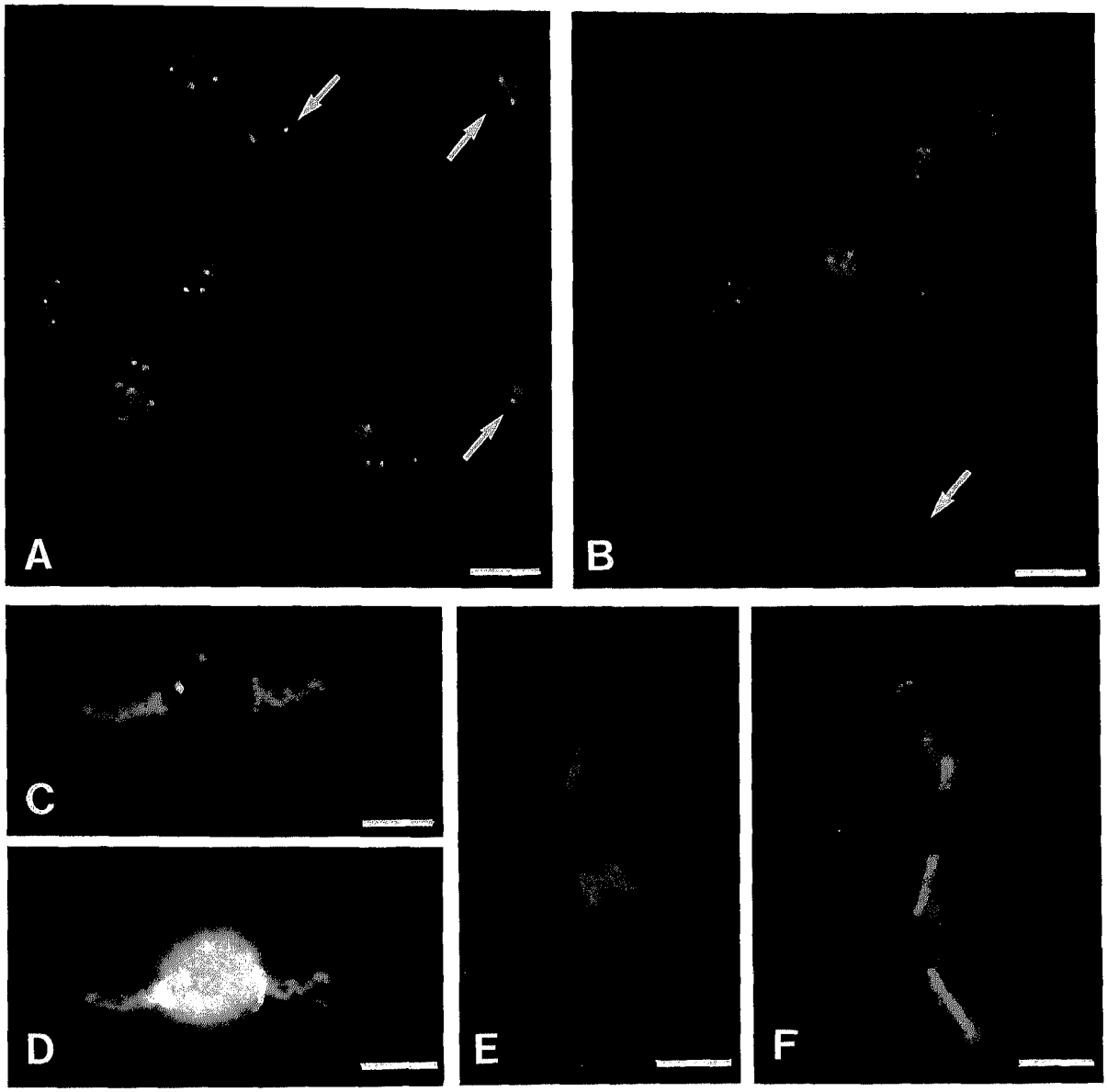

FIGURE 1 – Results of FISH with a centromeric probe for chromosome 7 and combination of FISH and ICC for cytokeratin 18. Nuclei from epithelial cells were isolated from colon adenomas. *(a, b)* Nuclei with trisomy and disomy (arrows) of chromosome 7. *(c, d, e, f)* Combination of ICC for cytokeratin 18 (alkaline phosphatase/fast-red reaction) and FISH. Counterstaining for *a, b* with PI and for *d* with DAPI. Scale bars: *a, b, e, f*: 30 μm; *c, d*: 20 μm.

FISH reaction

The optimal procedure for proteolytic digestion of nuclei and cells with pepsin was first tuned with different concentrations of pepsin in 0.01 N HCl. A concentration of 100 μl/ml pepsin in 0.01 N HCl for 15 min at 37°C gave the best results. The standard procedure for FISH, as described above, was successfully applied to all suspensions. In all cases the protocol resulted in FISH signals that could be evaluated according to the criteria described before (Hopman *et al.*, 1988).

Evaluation of FISH results in normal colon

FISH on 10 samples of endoscopically and histologically normal-looking colon, present in tissue resections from patients with carcinoma, showed no aberration for chromosomes 1, 7, 17 or Y. Only one tissue sample from a male patient showed disomy for chromosome X.

Evaluation of FISH results in adenomas

Counting and evaluation of the FISH signals for chromosomes 1, 7, 17, X and Y (Table II), revealed at least one numerical chromosome aberration in 14 out of 35 adenomas (40%). In Table II we have listed only those cases in which a numerical chromosome aberration was detected. The percentage of aberrant cells found within the samples summarized in this Table ranged from 15% to 70% [the average percentage of the cells with trisomy 7 was 32% (n = 14; SD 23); the average percentage of the cells considered as disomic (normal) for chromosome 7 was 4.5% (n = 21; SD 3.4)]. In 13 out of the 14 cases only a chromosome gain was detected. The most striking observation was that in 13 out of 14 cases showing genomic changes, a trisomy 7 was detected. Other changes mostly consisted of trisomy or tetrasomy of chromosomes 1 and 17, disomy, trisomy, and in one case even tetrasomy of chromo-

some X and disomy of chromosome Y. In only one case (case 21) did we observe a loss of chromosome 1. Figure 1 (*a, b*) depicts some typical examples of a trisomy 7.

A representive schematic overview of the distribution pattern of the different chromosomes in the case of an adenoma with trisomy 7 in 70% of the nuclei (case 13) is shown in Figure 2*a*. An example of such a distribution in an adenoma with numerical chromosome aberrations detected for all the investigated chromosomes (case 18d) is shown in Figure 2*b*. The numerical chromosome aberrations detected in case 18d comprised a trisomy of chromosomes 1, 7 and 17 and a disomy of chromosomes X and Y.

Combined ICC and FISH

On the basis of the isolation procedure (resulting in about 80% epithelial cells) and the percentage of trisomy for chromosome 7 as detected by FISH (percentage range 15–70%), it appeared likely that the cells with this aberration are epithelial in nature. Final proof, however, was obtained by a combination of ICC for cytokeratin 18 and FISH with a centromeric probe for chromosome 7. The results of this experiment (Fig. 1, *c–f*) clearly show trisomy 7 in epithelial cells, whereas non-epithelial cytokeratin-18-negative cells were generally diploid for chromosome 7 (not shown).

Correlation of chromosome aberrations with histopathological parameters

Four (30%) of the adenomas ≤ 1 cm (n = 13) and 9 (45%) of the adenomas > 1 cm (n = 20) appeared to be aberrant for at least one of the investigated chromosomes.

Aberrations were detected in 11 (39%) of the tubular adenomas, 2 (40%) of the tubulovillous adenomas and 1 (50%) villous adenoma. In addition, 3 hyperplastic polyps were investigated, in which no aberration was detected. Of the investigated adenomas 40% (n = 2) with a mild degree of dysplasia, 38% (n = 9) with moderate dysplasia and 50% (n = 3) with severe dysplasia showed at least one chromosomal aberration.

Heterogeneity within adenomas of one patient

In a 55-year-old male patient (case 18) 7 distinct adenomas were detected, varying in size from 4 to 33 mm. Six of these 7 adenomas were of a tubular phenotype and one was classified as a tubulovillous adenoma. The degree of dysplasia varied from moderate to severe. In 4 out of these 7 adenomas numerical chromosome aberrations were detected. In 2 samples (cases 18a and 18f in Table II) the numerical aberration found was a trisomy 7. In the third sample (case 18d) all the investigated chromosomes were aberrant and in the fourth sample (case 18b) trisomy of both chromosomes 7 and 17 was found.

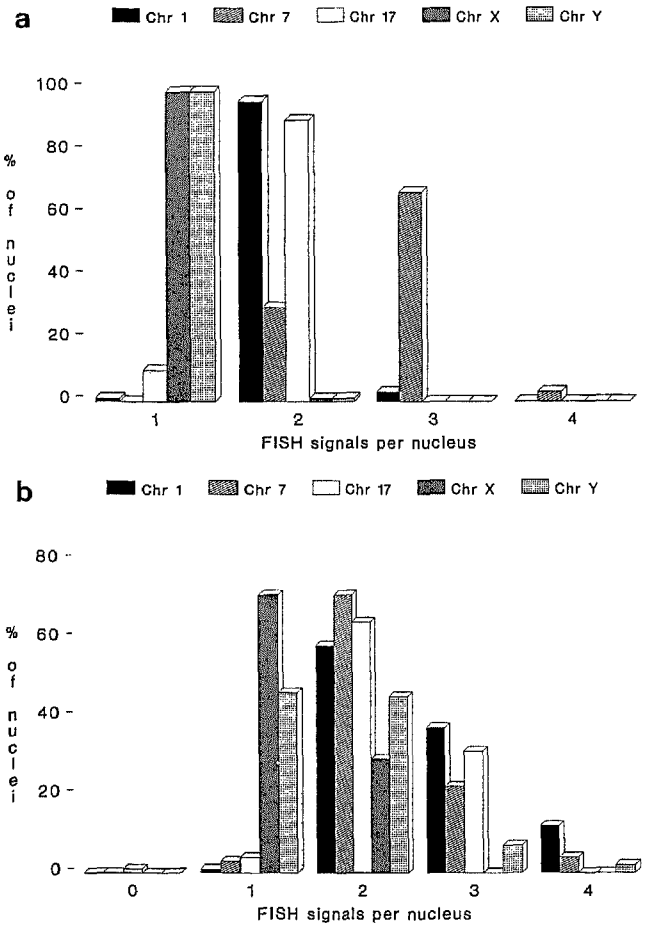

FIGURE 2 – Number of FISH signals per nucleus for the centromeric probes for chromosomes 1, 7, 17, X and Y in case 13 *(a)* showing trisomy 7 in about 70% of the nuclei, and in case 18d *(b)* showing numerous chromosomal aberrations.

DISCUSSION

Most studies on chromosomal abnormalities of solid tumors are performed by karyotyping of (primary) cell cultures. To avoid artefacts caused by selective advantage during *in vitro* cell culture, we used interphase cytogenetics with FISH for quantificative studies of numerical chromosome aberrations. Because of the dense tissue structure of the colon epithelium and of cells in the crypts of the adenomas, we could not properly analyse nuclei in paraffin sections or frozen sections used for FISH. Therefore, we were forced to use suspensions of cell nuclei obtained from fresh adenoma tissue.

In this study we detected no clear correlation between size, growth pattern and degree of dysplasia, on the one hand, and numerical chromosome aberrations on the other (Tables I, II). However, we must consider that the number of tubulovillous and villous adenomas, as well as adenomas with a mild or severe degree of dysplasia, is much smaller than the number of tubular adenomas and adenomas with a moderate degree of dysplasia. In the case with multiple adenomas (case 18a–g in Table I), 4 out of 7 samples showed chromosome aberrations (Table II) and in 2 out of 7 adenomas a trisomy of chromosome 7 was found as the sole aberration. In this case the smallest adenoma showed this feature, indicating that occurrence of trisomy of chromosome 7 is independent of the size of these lesions.

A numerical loss of chromosome 17, which is considered to be a late event in the tumorigenesis of colon adenocarcinomas (Fearon and Vogelstein, 1990), was not found in the adenomas.

In this study, 40% of the colonic adenomas were aberrant for the investigated chromosomes. In DNA flow cytometry studies about 30% of the adenomas were aneuploid (Giaretti *et al.*, 1988). Both results indicate that a fairly high percentage of colonic adenomas is aneuploid.

Our study points to 2 conclusions, that need further discussion. Firstly, we conclude that trisomy of chromosome 7 is a frequent aberration in colon adenomas; it occurred in 13 out of our 35 cases. In 6 of these, trisomy 7 was the sole numerical aberration detected after screening with the 5 chromosome probes. The finding of trisomy 7 in 40% of colorectal adenomas indicates that this aberration may be involved in the multi-step process of tumorigenesis that leads to colorectal cancer (Fearon and Vogelstein, 1990). This model is based on the detection of multiple allelic losses in colorectal tumors. Gain of genes may be another factor in this model, which describes the multistep process of malignant transformation in colon,

and one candidate gene that is located on chromosome 7 and may be involved in this process, is the EGFR receptor gene. In this respect the cytogenetic study of Muleris *et al.* (1990) in 100 colorectal adenocarcinomas is of importance. These authors reported 22 tumors in which most of the anomalies represented chromosome gains, and therefore these lesions were called "trisomic tumors". The most frequently occurring change in these tumors was a trisomy 7, while in this cytogenetic class of "trisomic tumors" no loss of either chromosome 17p or 18q was observed. Although this does not exclude mutations on both chromosomes, one might suppose trisomy 7 to be part of a different tumorigenic pathway.

The observation of trisomy 7 in non-malignant tissues, as reported in the literature (Bardi *et al.*, 1991; Heim *et al.*, 1989; Lee *et al.*, 1987; Limon *et al.*, 1990; Elfving *et al.*, 1990; Casalone *et al.*, 1992; Dal Cin *et al.*, 1992; Longy *et al.*, 1993) had questioned the significance of this aberration as an event related to neoplasia. In some studies (Heim *et al.*, 1989; Lee *et al.*, 1987; Limon *et al.*, 1990; Elfving *et al.*, 1990; Casalone *et al.*, 1992; Dal Cin *et al.*, 1992) trisomy 7 was found in normal brain, lung and kidney tissue, but all tissue samples used for these studies were taken from tumor-bearing organs. Bardi *et al.* (1991) concluded from a study on colorectal adenocarcinomas that trisomy 7 could be found in fibroblast-like cells, but on the other hand Williams *et al.* (1992) found trisomy 7 in epithelial cells. However, these studies were performed in tissue cultures of colon adenocarcinomas and it is well known that epithelial cells can assume a fibroblastoid phenotype under culture conditions (Greenburg and Hay, 1988). In a study by Arnoldus *et al.* (1991) on normal human brain tissue free of cancer, no trisomy 7 was found. Further evidence for a role of this aberration in neoplastic growth comes from a study of head-and-neck cancer (Voravud *et al.*, 1993) in which the frequency of cells with polysomy of chromosomes 7 and 17 increased as the tissue passed from histologically normal epithelium to cancer. In normal control oral epithelium from individuals free of cancer, no chromosome polysomy was found.

A second conclusion that can be drawn from our study is that trisomy 7 in these adenomas is found in the epithelial neoplastic component. This conclusion is based on the fact that the method of isolating the epithelial cells yields a suspension that contains at least 80% epithelial cells and about 20% stromal and inflammatory cells. Other strong evidence for this conclusion comes from the combination of cytokeratin immunocytochemistry and FISH. We found no trisomy 7, either in hyperplastic polyps, or in 10 samples of normal colonic epithelium.

We conclude that, for future studies, the combination of immunocytochemistry and FISH will become a valuable method of examining the correlation between genetic and phenotypic characteristics. In order to determine the significance of trisomy 7 as a neoplasia-related event in the colon, our next step will be the investigation of chromosome aberrations in tissue samples of normal mucosa, adenoma and adenocarcinoma all taken from the same patient.

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